Miniaturized bioassays for high-resolution effect-directed analysis of the aquatic environment

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Chapter 5

High-resolution effect-directed analysis of genotoxic and endocrine disrupting compounds in aquatic extracts

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Abstract

Effect-directed analysis (EDA) is a promising approach for the detection and identification of mutagenic and endocrine disruptive compounds during routine quality control of the drinking water production process. However, for routine toxicity assessment of multiple toxicological endpoints, current EDA is considered labor intensive and time consuming. To achieve faster EDA and identification, a high-throughput (HT) EDA platform, coupling a downscaled luminescent Ames and cell-based reporter gene assays with a high-resolution fraction collector and UPLC-QTOF MS, was developed. The applicability of the HT-EDA platform in the analysis of aquatic samples was demonstrated by analysis of extracts from WWTP influent, effluent and surface water. Downscaled assays allowed detection of mutagenicity and androgen, estrogen and glucocorticoid agonism following high-resolution fractionation in 228 fractions. From 8 tentatively identified masses, identified through non-targeted analysis, 2 masses were further investigated and chemically and biologically confirmed as mutagen 1,2,3-benzotriazole and androgen androstenedione. The compatibility of the high-throughput EDA platform with analysis of water samples and the incorporation of mutagenic and endocrine disruption end points allow for future application in routine monitoring in drinking water quality control and improved identification of (emerging) mutagens and endocrine disruptors.

Introduction

Mutagenicity and endocrine disruption are both important end points in water quality control of the drinking water production process as well as in toxicological and environmental screening of drinking water sources. Mutagens, and endocrine disruptive chemicals (EDCs) like estrogens[1], androgens[2], glucocorticoids (GCCs) and their metabolites are emitted into the aquatic environment through urban or industrial wastewater[3], agricultural runoff[4], deposition after combustion (PAHs)[5]. In addition, transformation during activated sludge, UV/peroxide oxidation and ozonation stages in water treatment leads to the formation of steroid hormone (metabolite) degradation and byproducts[6,7] and mutagenic byproducts[8,9]. The ability to rapidly determine the presence and identity of (previously unknown) mutagens and EDCs in drinking, surface, ground and effluent water is essential in efforts to keep track of their release

into the environment and to assure their removal during the drinking water purification process.

Previous studies have used the Salmonella/microsome test (Ames test) and cell-based luciferase reporter gene assays in effect-directed analysis (EDA) for the identification of (novel) mutagens and EDCs in environmental samples, respectively[10–12]. Via EDA, compounds not analyzed by routine (chemical) analysis are identified based on their biological activity in bioassays. Bioactivity measured in sample fractions, collected following chromatographic separation, are correlated to accurate masses detected in parallel on a mass spectrometer. Via non-targeted analysis, corresponding structures of masses in active fractions can be identified. Reducing fraction complexity through high-resolution fractionation decreases the number of compounds per fraction. The number of masses to be identified will also decrease as a result, which leads to a faster and more focused identification.

This study focused on the improvement of the current EDA approach by increasing the throughput and the fractionation resolution to allow faster identification. To allow high-resolution EDA for mutagens and endocrine disruptive chemicals, four different bioassays were used to screen 228 collected fractions from a surface water, a wastewater influent and a wastewater effluent extract. The bioassays consisted of a recently developed downscaled luminescent variation to the Ames test[13], the recently downscaled androgen receptor (AR) (AR-EcoScreen)[14] and estrogen receptor (ER) (VM7Luc4E2)[15] reporter gene assays[16], and the glucocorticoid receptor (GR) GR-CALUX reporter gene assay[17]. In parallel to the fractionation, mass spectra were recorded. Masses at those retention times that corresponded with the bioactive fractions were analyzed. Differences in bioactive fractions between wastewater extracts were analyzed to determine masses from compounds that were degraded or formed during water treatment. Finally, a qualitative non-target screening was performed to (tentatively) identify bioactive compounds.

Materials and methods

Materials

Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) medium with glutamax, phenol-free DMEM/F-12 medium with L-glutamine, low glucose phenol-free

DMEM medium, and fetal bovine serum were obtained from Gibco (Eggenstein, Germany). Penicillin/streptomycin, hygromycin, zeocin, G418, ampicillin, kanamycin, ATP, co-enzyme A, biotin, histidine and methanol (Chromasolv) were obtained from Sigma (Zwijndrecht, The Netherlands). Luciferin was obtained from Promega (Fitchburg, WI, USA), DTT from Duchefa (Haarlem, The Netherlands), and Aroclor 1254 induced rat liver S9 fraction from MP Biomedicals (Santa Ana, USA). Water was purified on a Milli-Q Reference A+ purification system (Millipore, Bedford, MA, USA). Reference compounds used for validation of the downscaled test methods and candidate compounds for confirmation of hits were obtained from various suppliers (see Supporting Information Table S1) and were dissolved in DMSO (Acros, Geel, Belgium).

Cell culture conditions

AR-EcoScreen (CHO-K1), VM7Luc4E2 (formerly known as BG1Luc4E2) (MCF7 human breast carcinoma) and GR-CALUX (U2-OS) cells were maintained as described by Satoh et al.[14], Rogers and Denison[15], and Van der Linden et al.[18], respectively. Briefly, cells were cultured at 37 °C with 5% CO2 in DMEM/F-12 medium with 10% fetal bovine serum and 1% penicillin/streptomycin (AR-EcoScreen and VM7Luc4E2 cells) or DMEM/F-12 medium with 7.5% fetal bovine serum and 1% penicillin/streptomycin (GR-CALUX cells), further referred to as culture medium. Cells were sub-cultured twice per week.

Sample preparation

Water samples were collected from the river Meuse and at the influent and effluent stream of an activated sludge municipal wastewater treatment plant (WWTP) in The Netherlands serving a population equivalent of approximately 200 000. Samples were stored at -20 °C until extraction. Thawed samples were filtered (250 µm mesh). Prior to extraction, SPE Waters 500 mg 6 cc BEH cartridges were conditioned with 4 mL 50/50% (v/v) MeOH/ethylacetate and equilibrated with 4 mL H2O. Water samples from the river Meuse (1 L), WWTP influent (100 mL) and WWTP effluent (500 mL) were extracted in triplicate. Samples were applied to cartridges and compounds were eluted 3 times with 3 mL MeOH/ethylacetate. The river Meuse sample was processed as two 500 mL aliquots and extracts were combined. Extracts were evaporated under a gentle stream of nitrogen at 30 °C and redissolved in 500 µL 10% MeOH/H2O or 50 µL 100% DMSO to reach concentration factors of 200 and 2000 times (WWTP influent), 1000

and 10000 times (WWTP effluent), or 2000 and 20000 times (Meuse river), respectively. From each sample, one extract was diluted 1, 3, 10, 30, 100 and 300 times in DMSO as a serial dilution series for screening of the unfractionated sample for mutagenicity or (ant)agonistic activity on hormone receptors. The two methanol/water extracts from each sample were used for EDA and target analysis, respectively.

Downscaled luminescent Ames test

The downscaled Ames test was performed as previously described[13] with minor modifications. Briefly, TA98lux and TA100lux luminescent Salmonella strains were grown overnight and diluted in exposure medium consisting of minimal medium supplemented with D-biotin and L-histidine. Reactions were prepared in 100 µL volumes in 96-well plates (n = 1). To each well 2 µL sample in DMSO and 98 µL bacterial suspension was added with or without the addition of 3.4 µL rat liver S9-mix for generation of metabolites. Prepared reactions were transferred to white PP 384-well plates in five 10 µL aliquots. Bacterial suspension on 96- and 384-well plates were exposed for 120 min at 37°C. Before and after exposure, the cell density of the TA98 exposure was measured in the remaining (50 µL) reactions on the 96-well plate at OD595 and compared to the negative control cell density as measure for cytotoxicity. Cell density growth below 75% of the negative control density were considered cytotoxic. Exposed cells on the 384-well plate were supplemented with 50 µL reversion medium (minimal medium with D-biotin but without histidine) and incubated for 28 hours until measurement. In each extract screening experiment (n = 1), cells were exposed in the down-scaled format fivefold to 1, 3 and 10 time dilutions of each extract.

Cell-based reporter gene assay protocol

Cell-based luciferase reporter gene assays were performed in 384-well plates (downscaled format) as previously described[16]. Briefly, cultured cells were seeded on 384-well plates and exposed to 34 µL AR-, ER- or GR-assay medium (phenol red- and antibiotic-free medium with charcoal stripped FCS) with extract dissolved at a final concentration of 1 µL/mL for 24 hours. Prior to exposure, DMSO was added to VM7Luc4E2 assay medium to expose cells at a final DMSO concentration of 5 µL/mL. Exposed cells were lysed and luminescence output was measured by luminometer. GR-CALUX cells were handled using the identical downscaled procedure in 384-well plates but at cell seeding densities and in phenol red-free DMEM/F-12 assay media as

described by Van der Linden et al[18]. In each extract screening experiment (n = 1), cells were exposed in triplicate to a serial dilution series of the extracts and reference agonist DHT (AR), 17β-estradiol (E2) (ER) or dexamethasone (GR). In antagonism experiments cells were exposed to extracts or reference antagonists flutamide (AR), fulvestrant (ER) or mifepristone (GR) in the presence of 200 pM, 5 pM or 1 nM of the reference agonists, respectively.

LC-MS analysis and fractionation

Separation of sample extracts was performed on a Waters Acquity UPLC BEH C18 1.7 µm 2.1 x 150mm column using an Agilent infinity 1290 UPLC pump and autosampler. Extracts in methanol/water were injected (250 µL) at a flowrate of 400 µL/min in 99% mobile phase A (100% H2O) and 1% mobile phase B (100% MeOH). The solvent gradient increased to 80% mobile phase B over 20 minutes and was subsequently kept as such for 2 minutes. Post-column, the flow was split in a 9:1 ratio with 9 parts being diverted to the FractioMate fraction collector and 1 part to a microTOF II time-of-flight mass spectrometer (Bruker Daltonics, Billerica, MA, USA). The mass spectrometer was equipped with an ESI source set to negative of positive mode and scanned masses from 50 m/z to 3000 m/z at 10 Hz. Corona and capillary voltages were set to 500 and 4500 V respectively. Nebulizer pressure was kept at 2 bar and nitrogen drying gas flow was kept at 6 L/min. Fractions were collected in white PP 384-well plates (Greiner Bio-One) for the Ames test or transparent PS 384-well plates (Greiner Bio-One) for cell-based reporter gene assays.

Exposure of fractionated extracts

In each experiment (n = 1), sample fractions were collected in 384-well plates (228 fractions) filled with 4 µL 10% DMSO in milliQ water. Fractions were collected at 6 seconds (228 fractions) intervals. Collected fractions were dried in a Centrivap concentrator for 5 hours at 25°C under vacuum until dry.

Exposure for cell-based reporter assays were performed as previously described with minor modification. Briefly, dried fractions on plates used for cell-based reporter gene assays were redissolved for 10 minutes on an orbital shaker at 700 rpm after adding 12 µL VM7Luc4E2 assay medium (with 1.8% DMSO), which is the most minimal of the three assay media, to aid in dissolving of the compounds. To each fraction 50 µL VM7Luc4E2 assay medium was added to dilute the DMSO concentration to 0.1%. Assay medium on seeded cells was refreshed with 24 µL fresh assay medium

containing 0.56% DMSO for VM7Luc4E2 cells or 0% DMSO for AR-EcoScreen and GR-CALUX cells. Cells from each cell line were exposed in parallel to fractions from a single fraction collection (parallel exposure). From each redissolved fraction, 10 µL was transferred to the 24 µL fresh assay medium on seeded cells from each cell line. Exposure was performed at a final DMSO concentration of 0.1% (1 µL/mL) for AR-EcoScreen and GR-CALUX cells or 0.5% (5 µL/mL) for VM7Luc4E2 cells. In each experiment (n = 1), each fraction was analyzed in a single well on each reporter gene assay.

Prior to exposure of Salmonella strains in the Ames assay, TA98lux and TA100lux overnight cultures were diluted to 180 (TA98lux) or 45 (TA100lux) FAU in exposure medium. DMSO was added to a final concentration of 2% (20 µL/mL suspension). One part of each suspension was supplemented with 34 µL S9-mix per mL suspension for generation of metabolites (-S9 or +S9 exposure suspension). Separate positive controls were prepared in exposure suspension without DMSO by adding 20 µL/mL reference compound dissolved in DMSO to a final concentration of 10 µg/mL 4-NOPD (TA98lux), 0.25 µg/mL NF (TA100lux), 0.1 µg/mL 2-AA (TA98lux +S9) or 0.4 µg/mL 2-AA (TA100lux +S9) (positive control suspension). In each experiment (n = 1), 10 µL exposure suspension was added to each dried fraction and 6 negative control wells or positive control suspension to 6 positive control wells in the 384-well fraction collection plate. The outer two rows of the plates were filled with 50 µL MQ water, and plates were incubated for 120 minutes at 37°C (150 rpm). After the exposure, 50 µL reversion medium was added to each well to a total volume of 60 µL per incubation. Incubation plates were kept at 37°C for 28 hours and were measured without lid on a VarioSkan Flash luminometer (Thermo) preheated to 37°C.

Data analysis

Bioassay results were analyzed in Prism 5.04 (Graphpad Software Inc, San Diego, CA). Cell-based reporter gene assay dose response curves were fitted with a four parametric logistic function [Y=A+(B-A)/(1+(x/C)^D)], where A and B denote minimal and maximal response respectively, C is the EC50 or IC50, D is the hillslope and x represents the tested concentration. Unfractionated extracts were tested in a single experiment. Reporter gene assay responses were interpolated in the linear phase of dose response curves of the cell line specific reference hormone. Calculated concentrations from one or more dilutions were averaged and reported as equivalent

concentration in ng/L to the respective reference hormone. Measured responses of unfractionated extracts in the Ames test were scored as previously described[13] and reported as the number of revertants out of 5 possible revertants. Samples were regarded mutagenic when the response was above 2 revertants per 5 wells. Responses to fractions in the Ames test were reported as fold-induction compared to the response to the negative control response.

Identification and confirmation

Non-target analysis was performed on masses correlating with active fractions as previously described[16] with minor modifications. In brief, masses (with a signal-to-noise ratio above 30) were selected for identification when detected in active fractions and not detected in inactive fractions at the same retention time in multiple samples (Fig. 1). Molecular formulas were determined based on the exact mass and isotopic distribution. Corresponding structures were retrieved from PubChem and Chemspider databases. Partition coefficients (logP) were predicted for each retrieved structure with ALOGPS 2.1 software and retention time was calculated based on experimental retention time/logP correlation data. Exclusively structures with predicted retention times that corresponded with the observed retention time were selected. Candidate structures with a compound name or that were described in literature were manually selected from the remaining structures. Fragmentation patterns of candidate structures retrieved from the mzCloud database were matched with the recorded mass spectrum. Biological data for a candidate was retrieved from literature or from PubChem and ToxCast toxicological databases. Mutagenic activity was predicted with the ToxTree software (carcinogenicity and in vitro mutagenicity decision trees). Candidates selected for confirmation were tested on the LC/MS to confirm retention time and mass spectrum. Bioactivity was confirmed on the respective bioassay.

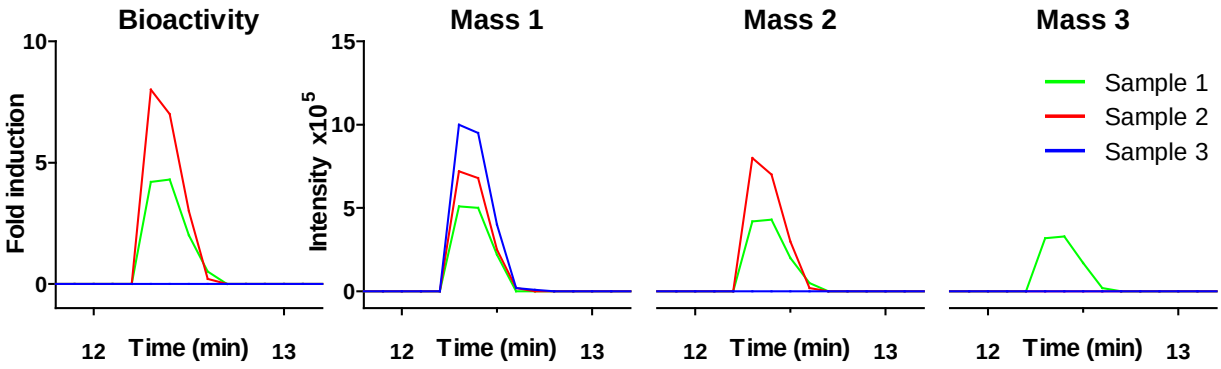

Figure 1. *Schematic representation of mass selection criteria for identification. Masses detected in combination with bioactivity at a specific retention time in multiple samples are selected (mass 2). Masses detected at the same retention time in non-active sample fractions of other samples (mass 1) or masses not detected at the same retention time in active sample fractions of other samples (mass 3) are filtered out.*

Results and Discussion

Mutagenic, androgenic, estrogenic and glucocorticoid and mutagenic potency of unfractionated surface water and wastewater extracts

The mutagenic and the agonistic and antagonistic potency of three water samples was determined by testing the unfractionated extracts on the luminescent Ames and AR, ER and GR reporter gene assays, respectively. Mutagenicity was detected in WWTP influent and effluent (TA98 and TA100) and river Meuse (TA98) extracts diluted 1, 3 or 10 times (Table 1). Mutagenicity was detected under all conditions in all samples except in river Meuse by TA100 and WWTP effluent by TA98+S9. The absence of measureable mutagenicity may be explained by the absence of base-pair substitution mutagens or the presence cytotoxicity respectively. All extracts were selected for further investigation after fractionation.

AR, ER and GR-agonism was observed in the WWTP influent, WWTP effluent and river Meuse extracts (ranked from the strongest to weakest response) (Table 2). Antagonism was observed in the GR (at 32.2, 303 and 844 ng mifepristone eq/L in Meuse, WWTP influent and WWTP effluent, respectively), but not in AR or ER assays. GR agonism and antagonism were both detected in WWTP in- and effluent extracts. This may indicate the presence of GR agonists less potent compared to dexamethasone, present during the antagonistic assay. While the agonists produce a response in the agonist assay, dexamethasone can be displaced in the antagonistic assay which results in reduced induction registered as antagonism. The antagonistic

response was not further investigated in fractionated samples. Bioactivity of unfractionated extracts was determined in a single experiment as a quick semi-quantitative screening method. To further increase throughput during sample selection for EDA, screening can be limited to 1 or 2 dilutions combined with a threshold induction for rapid determination of bioactive samples.

Table 1. *Mutagenicity (n = 1) expressed as number of revertants wells out of 5 possible revertants from separate exposures*

	TA98	TA98+S9	TA100	TA100+S9	Cytotox.
River Meuse	5	4	0	3	+
3x dilution	5	5	0	1	-
10x dilution	5	5	0	0	-
WWTP Influent	4	4	4	5	+
3x dilution	4	0	2	5	+
10x dilution	5	2	0	5	+
WWTP Effluent	1	1	4	5	+
3x dilution	4	1	2	5	+
10x dilution	4	1	0	3	-
Positive control	4	1	4	4	
Negative control	0	0	0	0	

Table 2. *Receptor activity (n = 1) expressed as the mean reference hormone equivalent concentration measured in one or multiple dilutions ± standard deviation*

	AR-agonism ng DHT eq/L	**ER-agonism** ng E2 eq/L	**GR-agonism** ng Dex eq/L
River Meuse	0.20	0.03 ± 0.03	Not detected
WWTP Influent	390 ± 61.6	9.80 ± 1.25	187 ± 22.4
WWTP Effluent	4.40 ± 0.89	0.43 ± 0.20	90.7 ± 54.9

Application high-resolution EDA to surface and wastewater extracts

Water extracts from the River Meuse and WWTP influent and effluent in The Netherlands were fractionated using UPLC and 228 collected fractions were tested in the downscaled luminescent Ames mutagenicity test (Fig. 2) and AR, ER and GR reporter gene assays (Fig. 3). Mutagenicity and AR, ER and GR agonism measured in unfractionated extracts could also be found in collected fractions. GR antagonism was not further investigated. Collected fractions from a single fractionation plate of each extract were analyzed on the three (AR, ER and GR) reporter gene assays exposed in parallel (parallel exposure). The parallel exposure method previously described allowed exposure of 8 assays in parallel[16] compared to 3 assays in the current study. Dissolving fractions in a smaller volume, to expose a smaller number of assays,

increased exposure concentrations in each separate reporter gene assay. The increased concentrations, however, did not lead to oversaturation of the responses in the reporter gene assays. For the Ames test, a separate injection followed by fraction collection was performed for each test condition (TA98, TA100, TA98+S9 and TA100+S9) (Fig. 2), given the lower sensitivity of the Ames test (micromolar concentrations range) compared to the reporter gene assays (picomolar concentrations range). Compared to the response in unfractionated extracts reported as number of revertants, responses in fractions could be reported by their induction factor. Induction factors in unfractionated extracts were often low (TA98 <2 and TA100 <3; data not shown) and revertants did not grow in all wells (table 1). The higher induction factors in fractions may be explained by (1) the higher concentration of the compound in each well and (2) the purity of the compound after chromatographic separation. Complex mixtures may contain compounds which inhibit the mutagenicity or limit the availability of a mutagen. Given the observed responses at the currently used concentrations, the downscaled luminescent Ames test seems to be compatible with high-resolution fractionated samples and applicable for HT-EDA.

A limited number of mutagens has been identified in the aquatic environment[19] and often found associated with suspended particulate matter (SPM)[20]. Mutagens present in filtered water samples are restricted to water soluble compounds which further limits the number of compounds responsible for the mutagenic activity in the unfractionated extracts. This is reflected by a limited number of bioactivity peaks in the collected fractions (Fig. 2). Furthermore, elution of mutagens at various retention times indicate the presence of different classes of compounds with varying structures. In contrast, the most potent activators of hormone receptors in reporter gene assays are natural and synthetic steroid hormones which share structural similarity and solubility with similar retention times. This results in peak clusters with poor separation (Fig. 3) compared to the good peak separation observed in the Ames chromatogram (Fig. 2). The majority of observed mutagenicity was present at 5 hot spots/peaks. Additionally, minor peak features consisting of weak (<2 fold induction) responses from multiple adjacent fractions were observed (Fig. 2B). These were most prominent in the response of TA98 in the presence of S9 to fractions of the WWTP effluent extract at 6.5, 8, 15 and 16.5 minutes (Fig. 2B). While not investigated, this may reveal the presence of additional mutagens in the effluent extract. The absence of a comparable response in the WWTP

influent extract may be explained by its 5 times lower concentration factor compared to that of the WWTP effluent extract. Further increase in the extract concentration factor or larger injection volumes on the LC column can increase compound concentrations in each fraction. This in turn may improve detection of relevant mutagens present at lower concentrations by the luminescent Ames test and allow their identification.

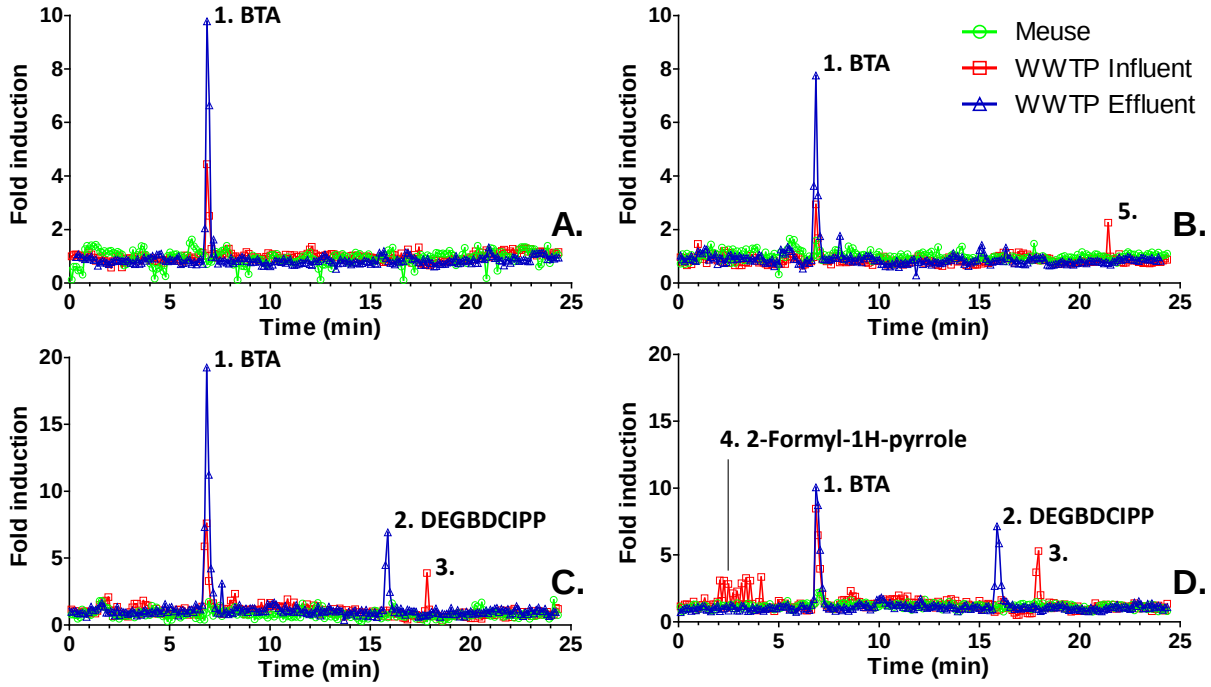

Figure 2. *Responses in the downscaled Ames to 228 fractions from water sample extracts in TA98 (panel A), TA98 +S9 (panel B), TA100 (panel C) and TA100 +S9 (panel D) (n = 1). Investigated peaks or features are numbered based on their order of induction and named with the (tentatively) identified structure.*

The majority of bioactivity measured in fractions by the AR-EcoScreen, VM7Luc4E2 and GR-CALUX corresponds with retention times of steroid hormones (Fig. S1 in the Supporting Information). Bioactivity in fractions from WWTP influent, however, was observed at earlier retention times compared to WWTP effluent. This may be explained by the presence of steroid hormone metabolites with increased water solubility present in WWTP influent[21]. Metabolites with increased water solubility like sulfate and glucuronide conjugates can, however, revert back to their unconjugated form through hydrolysis during water treatment[22]. A shift in bioactivity retention times between WWTP influent and effluent samples may therefore be explained by the formation of steroid hormone (metabolite) transformation products during water treatment. The HT-

EDA technique may be applied to investigate these changes in bioactivity patterns to track the removal or formation of bioactivity. This allows to determine water treatment efficiency and guide improvements to the treatment process.

Effective detection and identification of (novel) endocrine disruptors, however, is hindered by high concentrations of potent (synthetic) steroid hormones in aquatic samples which mask the response from often weaker (xenobiotic) EDCs. Due to similar log Kow values of various hormones, modifying LC conditions or further increasing the number of fractions may be insufficient to increase the bioassay peak separation.

Alternatively, potent (synthetic) hormones and their metabolites may be analyzed in fractions more effectively with a smaller MS/fraction collector split ratio (1:9 to 1:1). This may improve bioactivity peak separation when sample concentrations in fractions at the flank of a peak become too low to elicit a response. A smaller ratio also diverts more sample to the mass spectrometer which improves detection of masses during MS analysis. Identification of novel, xenobiotic, endocrine disruptors, however, may be more successful if focused on sources of potential EDCs that do not contain natural or synthetic hormones including plastics[23] or house dust[24].

Identification and Confirmation of Active Compounds

Masses present in active fractions were subjected to identification through non-targeted analysis. Resolution of the high-resolution fractionation method previously described[16], could be further increased from 192 to 228 fractions but did not lead to a reduced number of masses associated with each active fraction. The number of masses to identify could, however, be reduced further by selecting masses present in active fractions at the investigated retention time in multiple samples (Fig. 1). Alternatively, masses could be excluded when also detected at the investigated retention time in inactive fractions of a different sample. Poor ionization of bioactive compounds, however, can result in detection of masses in samples with high, but not in samples with low concentrations of the compound and can lead to false negatives. By determining the ratio between the bioactivity and MS intensity of the suspected mass, a prediction can be made of the mass spectrometer intensity corresponding with bioactivity in other samples. When, due to low bioactivity, the predicted MS intensity is below the detection limit, a mass can still be considered for identification.

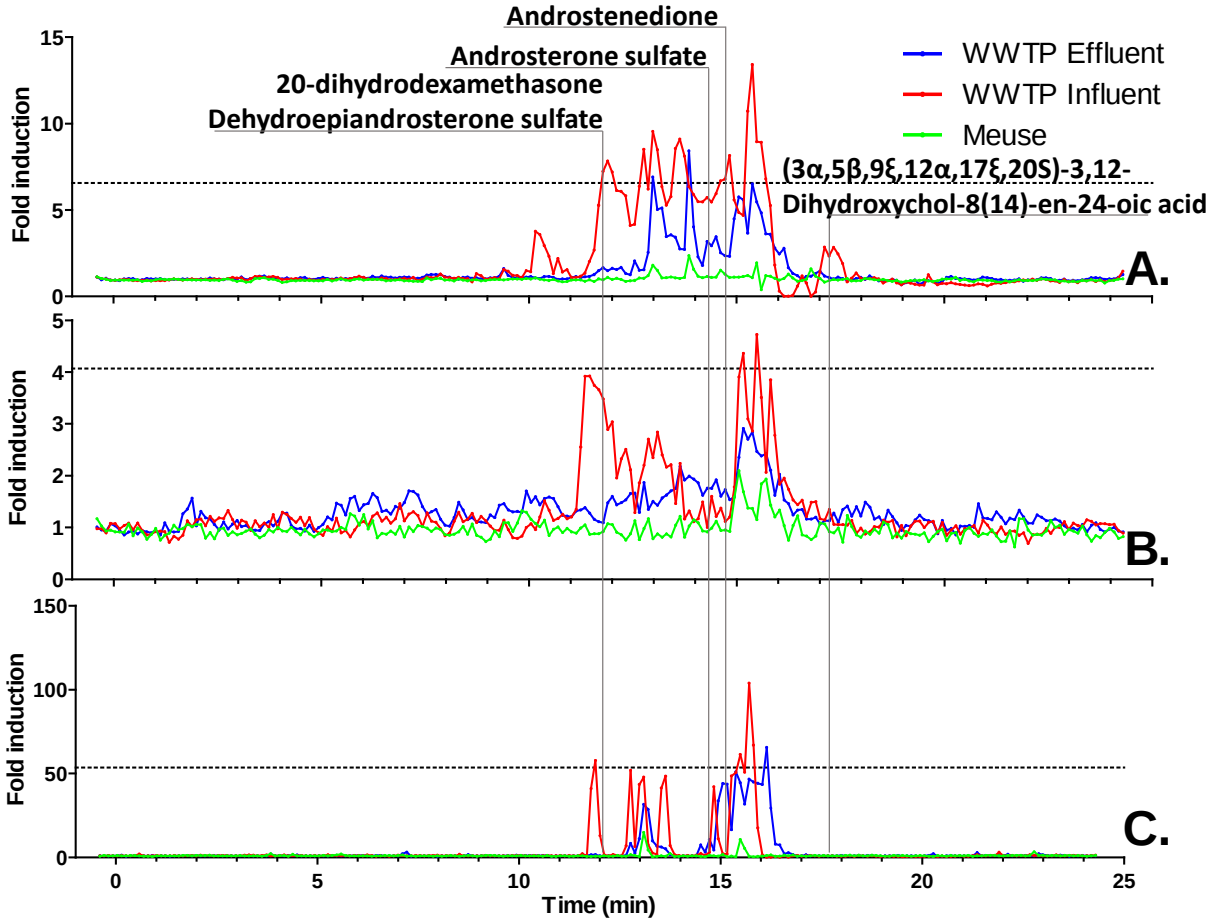

Figure 3. *Responses of the AR (panel A), ER (panel B) and GR (panel C) reporter gene assays to fractions (228) from river Meuse (green), WWTP influent (red) and WWTP effluent (blue) water sample extracts. Induction by reference hormones is indicated by the dotted line. Retention times of compounds (tentatively) identified are marked by a solid line.*

Structures were tentatively identified for 3 masses, out of 5 investigated peaks, observed in fractions producing a response in the luminescent Ames and for 5 masses observed in fractions producing a response in the AR-EcoScreen, VM7Luc4E2 or GR-CALUX. Identification of EDCs was focused on (synthetic) steroid hormones and their metabolites or transformation products. The (synthetic) steroid hormones are regarded as the receptor agonists responsible for the vast majority of observed bioactivity. Their metabolites and transformation products formed during water treatment, however, are the suspected cause of different peaks between responses in WWTP in- and effluent. From the candidates, 1 mutagen and 1 androgen were selected for analysis and subsequently confirmed as 1,2,3-benzotriazole (BTA) and androstenedione, respectively (Table 3; mass 2 and 4).

Table 3. *(Tentatively) identified masses and confirmed compounds*

Mass	Sample[1]	Source	RT (min)	m/z observed	MF	Err or [mDa]	mSigma	Potential structure	LogP	LogD (pH 7.4)	QSAR act.
Ames											
1	In	Pos	2.9	96.0441	C5H5NO	-0.3	4.5	2-Formyl-1H-pyrrole	0.64	0.65	Active
2	In	Pos/neg	6.7	120.0555	C6H5N3	0.1	15.5	1,2,3-Benzotriazole[2]	1.34	1.5	Inactive
3	Eff	Pos	15.8	571.0347	C16H32Cl4O9P2	0.5	15.4	DEGBDCIPP	3.04	n/a	Active
EDCs											
4	In	Pos	14.6	287.2002	C19H26O2	-0.4	17.8	Androstenedione[2]	2.9	2.9	Active AR
5	In	Pos	17.3	391.2836	C24H38O4	0.7	12.5	(3α,5β,9ξ,12α,17ξ,20S)-3,12-Dihydroxychol-8(14)-en-24-oic acid	4.37	4.28	Active AR
6	In/Eff	Pos	11.8	395.2232	C22H31FO5	0.4	14.6	20-dihydrodexamethasone	1.73	1.88	N.D.
7	In	Neg	11.7	367.1589	C19H28O5S	0.5	16.4	Dehydroepiandrosterone sulfate	3.71	-1.56	N.D.
8	In	Neg	14.4	369.1743	C19H30O5S	-0.1	19.3	Androsterone sulfate	4.02	-1.69	N.D.

1. (In)fluent or (Eff)luent 2. Confirmed

BTA (Fig. 2; peak 1) is a small polar molecule (log Kow 1.34) used as household and industrial corrosion inhibitor or drug precursor and was detected in Meuse water at low, and WWTP samples at high intensity. BTA was reported as Ames mutagen in a QSAR study[25] as well as in ToxTree QSAR analysis. Carcinogenicity was not determined in rodents, however, BTA is regarded as potential carcinogen and has been reported earlier in surface water and wastewater where it is poorly removed during wastewater treatment due to its solubility and resistance to degradation[26]. BTA was previously found active in TA98 and TA100 in the presence and absence of S9 activation in the regular agar-based Ames test[27,28]. BTA was also active in the newly developed luminescent Ames fluctuation test while no bioactivity was observed in the regular colorimetric Ames fluctuation test[13].

Diethylene glycol bis[di(2-chloroisopropyl) phosphate] (DEG-BDCIPP), a chlorinated phosphorus-based flame retardant, was tentatively identified as potential mutagen, but could not be acquired for further analysis. The mass was observed at 16 minutes in effluent (Fig. 2; peak 2) and identified based on the observed fragmentation pattern which corresponds with fragments reported by Matsukami et al.[29]. While no mutagenicity data of DEG-BDCIPP is known at the time of writing, chlorinated phosphorus-based flame retardant TDCPP has been found mutagenic in the TA100 Salmonella strain[30]. The bioactivity was undiminished following exposure in the presence of S9-mix, indicating resistance to metabolic degradation. However, the compound was not detected in the influent which might indicate that the observed mass is a breakdown product.

Additional major Ames bioactivity peaks 3, 4 and 5 were detected in WWTP influent at 18, 2 to 4, and 21 minutes respectively (Fig. 2). Peak cluster 4 was detected by TA100 in the presence of S9-mix and was tentatively identified as 2-Formyl-1H-pyrrole. Peak 3, detected by TA100 with increased bioactivity after exposure in the presence of S9-mix, and peak 5 detected by TA98 only in the presence of S9-mix could not be identified.

Androgen androstenedione, observed in WWTP influent and at 3.5-fold lower intensity in WWTP effluent, was selected as candidate compound and could be confirmed chemically based on retention time and mass spectrum in positive ESI mode. Masses from other routinely monitored hormones including estrone (E1), 17β-estradiol (E2), estriol (E3) and testosterone or their metabolites could, however, not be detected

despite the use of ESI in negative ionization mode to improve detection of hormones[21,31]. While masses were not detected, retention times of E2, E3, testosterone and BPA corresponded with bioactivity peaks which suggest their presence and activity (Fig. S1 in Supporting Information).

Fractions with bioactivity in the WWTP influent but with reduced or absent bioactivity in the WWTP effluent extract were analyzed to identify bioactive compounds which are formed or degraded during water treatment. Two (human) steroid hormone sulfate esters and two hydroxylated metabolites, predominantly or exclusively present in influent, were tentatively identified (Table 3; mass 5-8). Sulfate esters were readily detected with ESI in negative mode and accompanied by sulfate fragments. While sulfate conjugated steroid hormones are inactive forms, CHO-K1[32], MCF-7[33] and U2-OS[34] express steroid sulfatase (STS) which catalyzes the hydrolysis of sulfate steroids to their, active, unconjugated form. While endogenous STS expression levels are low, sufficient deconjugated steroid hormones may form during the 24 hour exposure time to generate a response. The absence of previously observed bioactivity and masses in effluent extract may be explained by deconjugation[7,35] or transformation of steroid hormone (metabolites) during water treatment[36]. While loss of bioactivity was mainly observed, fractions in ER (14 min) and GR (16 min) were increasingly active in effluent compared to influent. This may indicate the presence of compounds not released into or readily formed in WWTP influent water. However, no masses could be identified.

Filtering of masses observed in the same active fractions from different samples aided in selecting relevant masses for identification. This indicated that the identification process can be further improved by developing a database of masses associated with active fractions over a large number of samples. Separate databases will, however, be required for different chromatographic conditions optimized for the detection specific compound classes. Additional MS analysis in negative mode, under the same chromatographic conditions, further increased the number of detected masses. This, however, did not improve the detection of common hormones at the present concentrations. Analyzing samples in negative ionization mode with optimized eluents (i.e. at basic pH levels) may increase the ionization of compounds and improve their detection. However, a separate fractionation and bioassay analysis is required for each sample as retention times are shifted under different chromatographic conditions. This approach, which doubles the number of pipetting steps with each additional separate

fractionation, will be feasible with the introduction of automated liquid handling. In addition, routine EDA of environmental samples depends on processing of a large number of samples followed by rapid and complete identification. Automation in bioassay analysis and identification will therefore be a requirement for future optimization of the HT-EDA platform for application in routine analysis of (aquatic) samples.

Conclusion

Hyphenation of the down-scaled luminescent Ames test and cell-based reporter gene assays to a high-resolution fractionation setup enabled detection of mutagenicity and endocrine disruptive potency in sample fractions, respectively. Mutagenicity, and AR, ER and GR activity could be analyzed in 228 fractions collected from surface and WWTP influent/effluent water extracts and were detected in all samples. The identification process could be improved through selection of masses detected in active fractions at the same retention time over multiple extracts, reducing the number of masses to analyze. Eight compounds were tentatively identified as active compounds from which BTA and androstenedione were further investigated and confirmed as active mutagen and androgen respectively. Introduction of the down-scaled luminescent Ames test to the HT-EDA platform enables rapid detection of mutagenicity, in addition to endocrine disruption, at high resolution and allows future application in the detection and identification of (novel) (geno)toxicants during (routine) monitoring of the drinking water production process.

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Chapter 5 - Supporting information

Table S1. *List of reference compounds and candidates selected for confirmation of their identity*

Chemical name	Supplier	CAS No.
Reference hormones		
5α-dihydrotestosterone (DHT)	R&H	521-18-6
17β-Estradiol (17β-E2)	Sigma-Aldrich	50-28-2
Flutamide	Sigma-Aldrich	13311-84-7
Fulvestrant	Sigma-Aldrich	129453-61-8
Dexamethasone	Sigma-Aldrich	50-02-2
RU486 (Mifepristone)	Sigma-Aldrich	84371-65-3
Reference mutagens		
4-nitro-o-phenylenediamine (4-NOPD)	Sigma-Aldrich	99-56-9
Nitrofurantoin (NF)	Sigma-Aldrich	67-20-9
4-nitroquinoline-1-oxide (4-NQO)	Sigma-Aldrich	56-57-5
2-aminoanthracene (2-AA)	Sigma-Aldrich	613-13-8
Reference compounds and candidates		
17α-Estradiol (17α-E2)	Sigma-Aldrich	57-91-0
4-tert octylphenol	Sigma-Aldrich	140-66-9
Androstenedione	Sigma-Aldrich	63-05-8
Bisphenol A (BPA)	Fluka	80-05-7
1,2,3-Benzotriazole (BTA)	Janssen	95-14-7
Estriol (E3)	Sigma-Aldrich	50-27-1
Estrone (E1)	Sigma-Aldrich	53-16-7
Ethinylestradiol (EE)	Sigma-Aldrich	57-63-6
Testosterone	Sigma-Aldrich	58-22-0

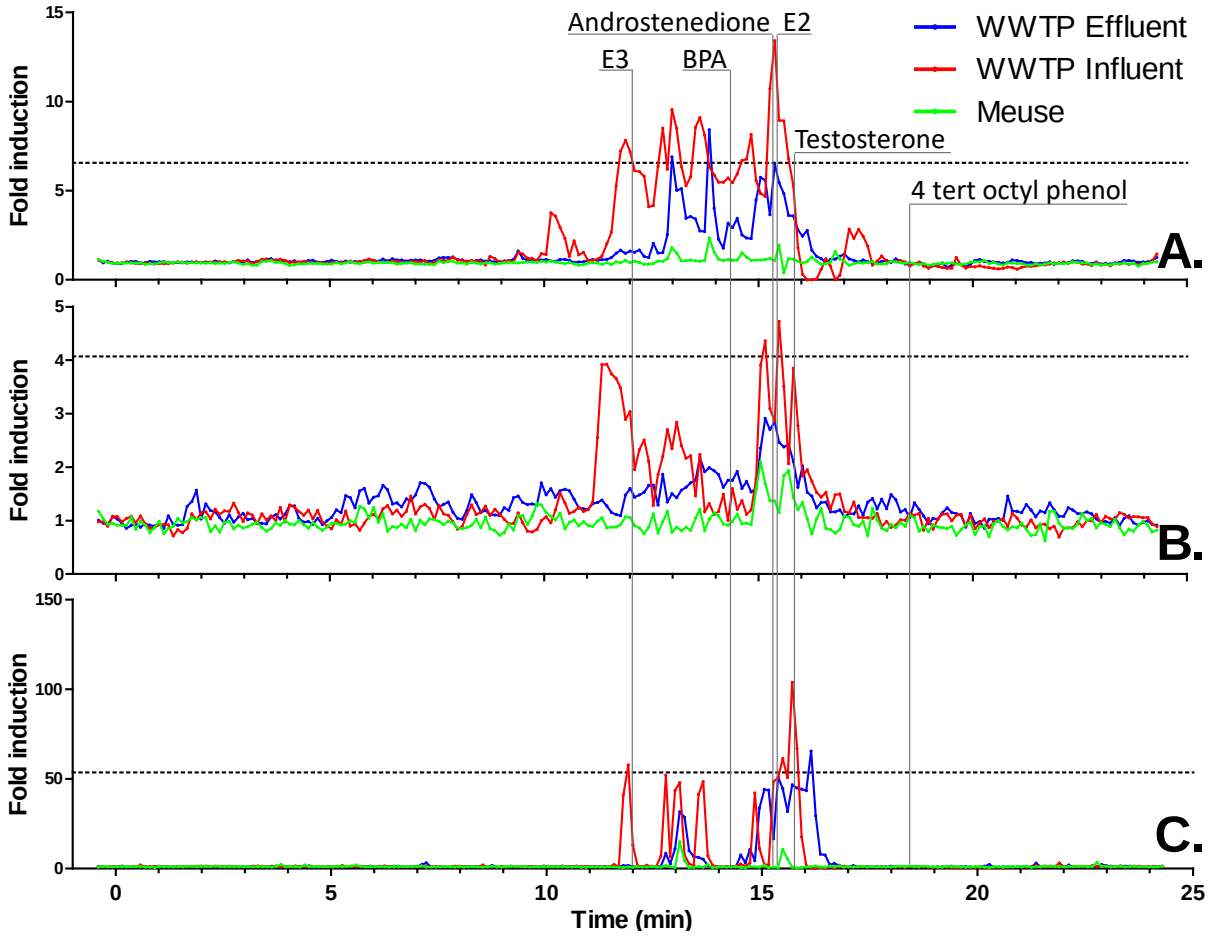

Figure S1. *Responses of the AR (panel A), ER (panel B) and GR (panel C) reporter gene assays to fractions (228) from river Meuse (green), WWTP influent (red) and WWTP effluent (blue) water sample extracts. Induction by reference hormones is indicated by the dotted line. Retention times of compounds analyzed with the same gradient are marked by a solid line.*